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Suppressor Gene

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13. ABSTRACT (Maximum 200 Words)
No effective therapy currently exists for androgen-independent, metastatic prostate cancer. Using an orthotopic animal model, we isolated androgen-sensitive and androgen-insensitive variants of the human LNCaP prostate cancer cell line. The latter display increased levels of BCL-2 and resistance to multiple triggers for apoptosis. Progress over the past year has demonstrated that the increased BCL-2 is linked to increased ligand-independent androgen receptor activity and increased serum-dependent activity of the transcription factor, NFkB. Parallel studies have evaluated the in vivo efficacy and toxicity of a novel drug, PS-341 (a proteasome inhibitor), that is capable of bypassing BCL-2-mediated resistance in vitro. The drug promoted extensive apoptosis in established orthotopic human PC-3 prostate tumors. Analysis of the mechanisms involved revealed direct effects on tumor cells as well as indirect effects on tumor vasculature. Future efforts will be directed at further dissecting the transcriptional control of apoptosis resistance in the androgen-independent prostate cancer cells. Further preclinical studies with PS-341 will include evaluating its effects on p53-positive (LNCaP-Pro5) orthotopic tumors and in combination with other chemotherapeutic agents.

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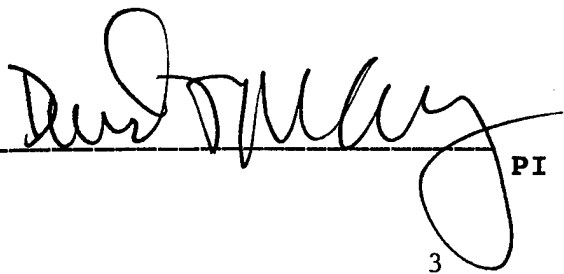
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Manuscript, "Immunofluorescence double labeling of apoptotic endothelial cells in situ: A biomarker for angiogenesis inhibition." D.W. Davis et al, submitted for publication (Cancer Research).

Abstract, "PS-341: A new agent with activity in prostate and pancreatic cancer." AACR-NCI-EORTC joint meeting, Molecular Targets and Cancer Therapeutics, Washington, D.C., November 16-19, 1999.

5. INTRODUCTION

Prostate cancer is the most common malignancy and the second leading cause of cancer-related deaths among men in the United States (1). Despite increased awareness of the disease and improved methods for early detection, a large proportion of patients die of disseminated cancer that is resistant to conventional therapies (2-4). Most of these therapies involve androgen ablation, which is at best palliative and which leads to rapid emergence of androgen-insensitive tumors in a majority of patients by 2 to 3 years posttherapy (5). Conventional chemotherapeutic agents exhibit little activity against androgen-insensitive tumors (4, 6). Therefore, understanding the biological and molecular mechanisms contributing to prostate cancer progression and drug resistance in androgen-independent disease is crucial to the development of more effective therapeutic regimens. Our preliminary work indicates that increased expression of the anti-apoptotic protein, BCL-2, is tightly coupled to androgen independence in a panel of human cell lines selected for increased metastasis in an orthotopic animal model (7). In addition, we obtained evidence that a novel cancer chemotherapeutic agent and proteasome inhibitor, PS-341, can bypass BCL-2-mediated cell death resistance in model cell lines in vitro. Therefore, this project's parallel goals were to (1) identify the molecular mechanisms involved in the increased BCL-2 expression observed in the androgen-independent cells, and (2) evaluate PS-341's efficacy against androgen-independent, orthotopic human prostate tumors in vivo. We have made substantial progress toward completing both of these goals.

6. BODY

Control of BCL-2 expression in androgen-independent cells. In our preliminary work we selected metastatic variants of the androgen-sensitive human LNCaP prostate adenocarcinoma cell line by orthotopic "recycling" in nude mice (8). The cell lines in our panel are LNCaP-Pro1, -Pro3, Pro-5 (selected sequentially for growth within the prostate), and -LN1, -LN2, -LN3, and -LN4 (selected for metastasis to the lymph node). Over the past year we have confirmed that the highly metastatic LNCaP-LN3 and -LN4 cells are resistant to thapsigargin and doxorubicin-induced apoptosis and that they express elevated levels of BCL-2. We obtained chloramphenicol acetyltransferase (CAT)-based full length and deletion constructs for the BCL-2 promoter from a colleague (Dr. John Reed, Burnham Institute). We had planned to use them to determine whether or not the increased BCL-2 expression noted in the LN3 and LN4 cells could be attributed to activation of particular transcription factors. However, our initial results with the full length BCL-2 construct were highly variable, most likely due to differences in transfection efficiency that are difficult to control for with CAT-based vectors. We have therefore obtained newer, luciferase-based BCL-2 promoter expression constructs from another colleague (Dr. Tim McDonnell at our own institution) and are repeating these experiments now. (The luciferase system includes a control vector that constitutively produces a variant luciferase protein that generates a product distinct from the test luciferase protein, allowing transfection efficiency to be monitored by simultaneous measurement of the two luminescence profiles. We are now using this system for all of our promoter work.)

In spite of the setback caused by our problems with the BCL-2 promoter constructs, our parallel efforts have established a novel connection between BCL-2 and androgen receptor activity that will most likely be highly relevant to the question of how BCL-2 expression is controlled in the LN3 and LN4 cells. Characterization of the properties of our cell lines revealed a direct correlation between increased metastatic potential and increased androgen independence as measured by growth of tumors in castrated mice (8) or by growth of the cells in androgen-depleted medium in vitro (Figure 1). Further study demonstrated that basal and ligand-independent androgen receptor activity was also increased in the metastatic variants (Figure 2). Thus, we speculate that ligand-independent androgen receptor activation could be responsible for the increased expression of BCL-2, and experiments with androgen receptor antagonists, a dominant negative androgen receptor construct, and the new BCL-2 promoter expression constructs are ongoing to directly test this hypothesis. Notably, our colleague (Dr. Tim McDonnell) has identified two consensus androgen response elements in the BCL-2 promoter that could be responsible for this effect. However, an alternative explanation is that BCL-2 is driving ligand-independent androgen receptor activation. To test this possibility, we generated

transfectants of the androgen-sensitive, non-metastatic LNCaP-Pro5 cell line that constitutively express levels of BCL-2 comparable to those observed in the metastatic LNCaP-LN3 cells. Strikingly, these transfectants also demonstrate appreciable ligand-independent activation of the androgen receptor (Figure 3). Therefore, we now suspect that BCL-2 may be promoting androgen receptor function as well. In separate studies conducted with our colleague, Dr. Curtis Pettaway, we are attempting to define the mechanisms (i.e. increased androgen receptor coactivator binding, alterations in androgen receptor chaperone proteins, alterations in intracellular localization) underlying the effects of BCL-2 on the androgen receptor.
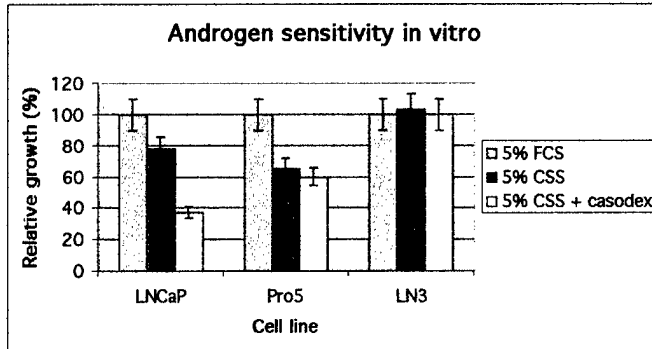

Figure 1: Androgen sensitivity of LNCaP-derived cell lines in vitro. Cells were growth in complete medium containing 5% normal serum (FCS), 5% charcoal-stripped serum(CSS), or 5% CSS plus 10 µM bicalutamide (casodex, an androgen receptor antagonist), and cell growth was determined at 96 h by direct cell counting. Mean ± S.D., n = 3.
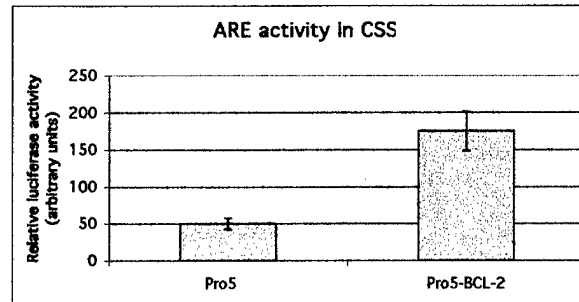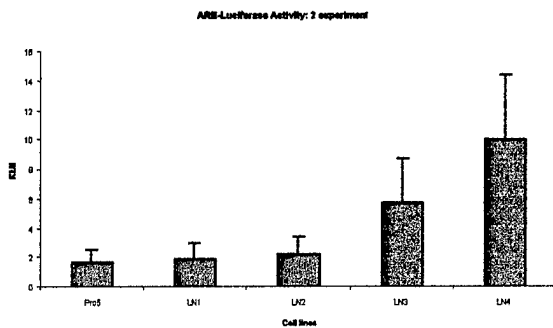

Figure 2 (left): Ligand-independent androgen receptor activity in the LNCaP panel. Cells were transfected with a promoter-reporter construct (obtained from Dr. Guido Jenster, M.D. Anderson Cancer Center) containing 3 tandem androgen-response elements (ARE's) driving luciferase expression. Transfection efficiency was controlled for in parallel by cotransfection of another luciferase construct driven by a constitutive (SV40 or CMV) viral promoter. Mean ± S.D., n = 3.

Figure 3 (right): Increased ligand-independent androgen receptor activity in BCL-2 transfectants. Pro5 or Pro5-BCL-2.3 cells were transfected with the reporter construct described in Figure 2, and promoter activity was evaluated in charcoal-stripped serum. Mean ± S.D., n = 3.

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In a second line of investigation we are addressing the possibility that BCL-2 expression in metastatic cells is controlled by exogenous growth factor(s). We conducted a simple preliminary experiment to to address this possibility. LNCaP-LN3 cells expressing high levels of BCL-2 were cultured in charcoal-stripped serum (to deplete androgens) or in the absence of serum altogether (to remove all growth factors), and BCL-2 levels were determined by Western blotting (Figure 4). No differences were observed between the control cultures (complete medium) and cells grown in charcoal-stripped serum. However, BCL-2 levels were dramatically lowered in the cells grown in the absence of serum. No effect of serum withdrawal on cell viability was observed after up to 7 days of culture, demonstrating that the effect was not due to cell death.
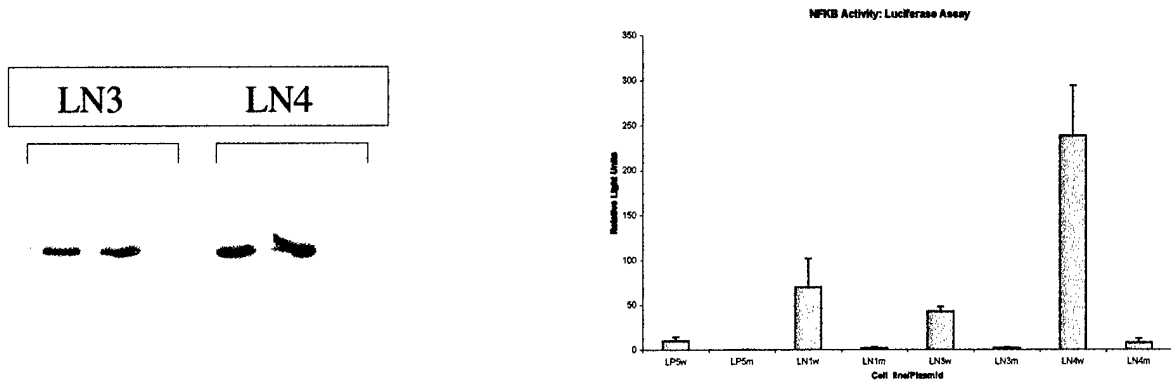

Figure 4 (left): Serum withdrawal suppresses BCL-2 protein levels in metastatic LNCaP derivatives. Cells were incubated in normal growth medium (10% serum, control lanes), in medium supplemented with 10% charcoal-stripped serum, or in serum-free medium for 48 h, and BCL-2 levels were evaluated in total cell extracts by Western blotting. Lanes 1-3, LNCaP-LN3; lanes 4-6, LNCaP-LN4. Lanes 1+4, control; lanes 2+5, charcoal-stripped serum; lanes 3+6, serum-free medium.

Figure 5 (right): Constitutive NFκB activity in LNCaP derivatives. Cells were transfected with an NFκB-specific promoter expression construct (containing the HIV promoter-enhancer NFκB site) for 24 h prior to evaluation of luciferase expression by luminometry; expression was controlled by cotransfection of a second (constitutive) luciferase construct. Mean values from triplicate samples.

One of the transcription factors activated by a variety of serum factors is NFκB, and our recent work in other model systems suggests that NFκB and BCL-2 functionally interact. Indeed, one of the most well known effects of proteasome inhibitors (see below) is their ability to block NFκB activation (9), and we wondered whether this might account for the high activity of proteasome inhibitors in the BCL-2 overexpressing, androgen-independent LNCaP-LN3 cells. Promoter-expression analysis of NFκB activity in our panel of cell lines confirmed that it increased as a function of metastatic potential (Figure 5). Ongoing studies with proteasome inhibitors and a dominant active polypeptide inhibitor of NFκB (IκBαM) are underway to further evaluate NFκB's role in maintaining high level BCL-2 expression. In the future we will also be interested in trying to determine the signal transduction pathway(s) responsible for serum-mediated BCL-2 upregulation in the LN3 cells, as well as identifying the growth and survival factor(s) involved.

Preclinical evaluation of PS-341, a proteasome inhibitor that can bypass BCL-2-mediated cell death resistance. PS-341 is a peptide boronate inhibitor of the proteasome developed by ProScript, Inc. (Cambridge, MA) for use in anti-cancer therapy. My laboratory has been concerned with defining the role of the proteasome in the regulation of apoptosis for several years, and in the course of these studies we made the fortuitous observation that PS-341 (and other proteasome inhibitors) kill LNCaP-Pro5 and LNCaP-LN3 cells equivalently

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in vitro. As noted above, this constrasts our experience with doxorubicin and other chemotherapeutic agents in this model system and suggests that PS-341 might have unique activity against advanced prostate tumors. This idea is consistent with the experience of Dr. Edward Sausville's drug development group at NCI, who determined that PS-341 had high and unique activity in their 60 cell line in vitro screen. Thus, the second major objective of the research outlined in this proposal was to evaluate PS-341 in our orthotopic animal model of human prostate cancer.

We began our study by conducting a combined toxicity/efficacy trial in mice bearing orthotopic PC-3M human prostate tumors. The PC-3M subline was isolated in our department by the orthotopic "recycling" strategy alluded to above and is highly metastatic to the lymph node and several distant organ sites. Tumorgenicity and metastatic frequency approach 100%, allowing for high reproducibility. In addition, ProScript had conducted a number of studies in subcutaneous tumors as part of their preclinical work leading to their IND submission, and they had reported up to 60% tumor regression in the PC-3 model.

In the first study (10 mice/group) we established the maximal tolerated dose (MTD) in our system. Drug was administered i.v. once weekly (tail vein injection) beginning at 1 week and continuing until week 4. Animals were dosed with 0.3, 0.6, 0.8, and 1 mg/kg PS-341, an escalation scheme designed with the help of ProScript and based on their own preclinical experience with the drug. No mortality was observed at either 0.3 or 0.6 mg/kg but reached 20% (2/10) at 0.8 mg/kg and 80% (8/10) at 1 mg/kg. In fact, no signs of toxicity of any kind were noted at necropsy of animals receiving 0.6 mg/kg PS-341. In contrast, animals receiving higher doses invariantly presented with gastrointestinal toxicity (i.e. diarrhea) prior to death. Therefore, 0.6 mg/kg was selected as the MTD for the subsequent studies.

We have conducted 4 separate in vivo studies with 0.6 mg/kg PS-341 on the PC-3M tumors, two using the once weekly schedule and two using a twice weekly schedule. The results of both schedules were very similar and are summarized in the representative experiment outlined in Table I. Grossly, PS-341 had no effect on tumor take, tumor weights, or lymph node metastasis. However, routine hematoxylin and eosin (H&E) staining of the primary tumors and lymph node metastases revealed extensive (33-50%) necrosis in all of the PS-341-treated tissues. Interestingly, this necrosis was confined to tumor-containing regions and was not observed in adjacent normal tissue. Quantification of apoptosis by terminal deoxynucleotidyl transferase uridine nick end labelling (TUNEL) revealed massive induction of apoptosis in all of the tumors from the treated groups as compared with very low levels of apoptosis in untreated controls (Figure 6). Thus, PS-341 does appear to have high activity at non-toxic doses.

Table I: Effects of PS-341 on standard parameters of tumorigenicity.

	Tumorigenicity	Mean weight	Median/range	LN metastasis	Distant metastasis
Control	12/12 (100%)	0.452±0.21	0.455/ 0.171-0.753	11/12 (92%)	1/12 (kidney & post ab wall)
PS-341	13/13 (100%)	0.482±0.19	0.491/0.120-0.829	11/13 (85%)	1/13 (post ab wall)

The pattern of central necrosis observed in the PS-341-treated tumors was highly suggestive of possible direct effects of the drug on tumor vasculature. Additionally, in parallel work we have found that hypoxia-induced activation of NFκB is required for expression of the proangiogenic factors including vascular endothelial growth factor (VEGF), and inhibition of NFκB is a well known effect of proteasome inhibitor action. We therefore stained the PS-341-treated tumors with an antibody to the endothelial cell antigen, CD31, in order to

quantify microvessel density. The results revealed a dose-dependent decrease in blood vessels (Figure 6), confirming that angiogenesis is a secondary target of drug action.
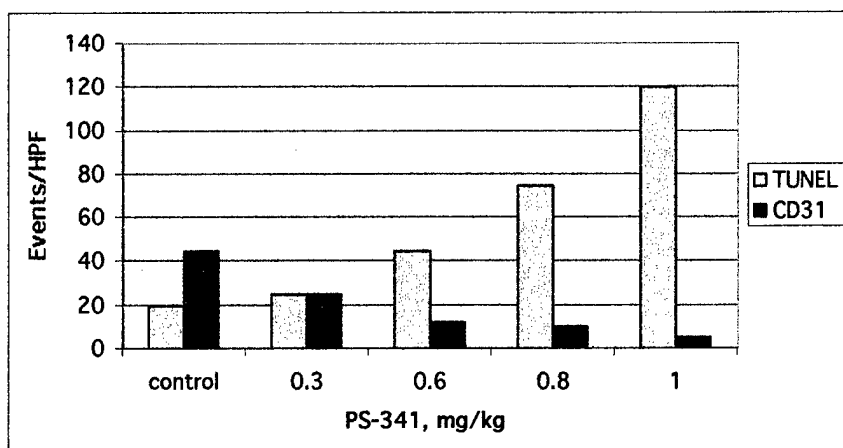

Figure 6: PS-341 induces apoptosis and inhibits angiogenesis in established PC-3M tumors. Animals were treated with 4 injections of 0.6 mg/kg PS-341 (i.v.) as described. Tumors were harvested two days after the last injection, and apoptosis was measured by TUNEL analysis. Right panel, control tumor; left panel, PS-341-treated tumor. Mean ± S.D., n = 5.

Although the PC-3M tumor model was ideal for the evaluation of drug toxicity and initial efficacy, it is an extremely aggressive tumor that is probably not an ideal target for proteasome inhibitor action. Another well known effect of proteasome inhibitors is their ability to stabilize the p53 tumor suppressor protein, which plays a critical role in several pathways of apoptosis. Over the past year we have directly examined the p53 status in our panel of LNCaP-derived cell lines by single strand conformation polymorphism (SSCP) analysis of exons 2, 4-9, and we have found no evidence for the presence of mutation(s). In contrast, the PC-3M line does not express wild-type p53. Therefore, even though we know that PS-341 can kill in a p53-independent fashion, we expect that activity will be even higher in a p53-positive tumor. In the second year of funding we will evaluate PS-341's effects against Pro5 and LN3 tumors to determine if this is indeed the case.

One of the most important motivating factors for conducting the preclinical work with PS-341 was that a Phase I clinical trial on the drug is now being conducted by Dr. Christos Papandreou in our Department of Genitourinary Medical Oncology. PS-341 is unlike any other drug evaluated to date, making predictions about toxicity very difficult. Currently, our trial has reached the predicted MTD (0.8 mg/m^2) with no signs of toxicity. Interestingly, 3 of the 15 patients evaluated to date have had clinical responses, with one patient exhibiting a very clear lymph node CR on CT analysis. Dose escalation in the protocol has been cautious (and mathematically insignificant) at 5%, and we have been involved in discussions with ProScript and members of the clinical team at M.D. Anderson to decide whether or not to modify the protocol and increase the dose escalation increments to 10%. Our preclinical toxicity data were invaluable in helping to convince the team that this would be possible but that a "pause" in escalation (i.e. additional patient accumulation) should be included in the trial when the 0.6 mg/kg equivalent dose is reached.

7. KEY RESEARCH ACCOMPLISHMENTS

❶ Ligand-independent AR activation is associated with metastatic progression
❷ BCL-2 directly promotes ligand-independent AR activation
❸ Serum factors (not androgen) contribute to increased BCL-2 expression
❹ The transcription factor NFκB is activated in the metastatic LNCaP variants
❺ Dose and schedule for PS-341 in animals bearing orthotopic prostate tumors have been determined
❻ PS-341 induces apoptosis and necrosis in established orthotopic prostate tumors
❼ Tumor vasculature is an unexpected, secondary target of PS-341 action

8. REPORTABLE OUTCOMES

❶ Preclinical evaluation of the proteasome inhibitor, PS-341, in an orthotopic model of human prostate cancer (manuscript in preparation)
❷ BCL-2 promotes ligand-independent activation of the androgen receptor in human prostate cancer cells (manuscript in preparation)
❸ PS-341: A new agent with activity in prostate and pancreatic cancer." AACR-NCI-EORTC, "Molecular Targets and Cancer Therapeutics," Washington, D.C, November 16-19, 1999 (see Appendix)
❹ Co-investigator, DOD Center Initiation Grant application (P.I.: Dr. Christopher Logothetis, M.D.), "The biology and therapy of advanced prostate cancer," 1999.
❺ Established a method for quantifying endothelial cell death in tumors in situ (see Appendix).

9. CONCLUSIONS

Successful progress has been made towards addressing both major objectives of this proposal. In the first part of the project we have identified a novel interaction between BCL-2 and the androgen receptor that could help to explain why androgen receptor antagonists lose their efficacy during tumor progression. It appears that BCL-2 expression (and most likely growth factors) "substitute" for ligand in activating the androgen receptor. In theory, this unorthodox AR activation could result in a global, atypical pattern of AR-mediated gene expression that may serve as a critical factor in the metastatic cascade. Understanding this interaction will most likely prove critical to understanding overall progression in this disease. New strategies for targeting BCL-2 and/or the AR by molecular means are emerging and could be tested in our models. We are optimistic that in our second year of support, new mechanisms involved in the maintenance of BCL-2 expression in metastatic cells will be identified as well.

Although its lack of effects on standard measures of tumorigenicity was initially discouraging, further analysis of PS-341's effects on established human prostate tumors provide new enthusiasm for its use in androgen-independent, metastatic disease. At levels of the drug that produce no visible toxicity in mice (0.6 mg/kg), PS-341 promotes extensive apoptosis leading to central necrosis in all tumors and lymph node metastases, effects that appear to be due to both direct effects on tumor cells and also to indirect induction of apoptosis in the tumor vasculature. Further study of PS-341's effects on less aggressive (hormone-responsive) tumors is indicated, and pending the outcome of these experiments, combination therapy with agents that act via proteasome-sensitive mechanisms (i.e. via p53, NFκB) should be evaluated. Preliminary results in a Phase I clinical trial being conducted at our institution further bolster enthusiasm for this drug.

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Immunofluorescence Double Labeling of Apoptotic Endothelial Cells *in situ*: A

Biomarker for Angiogenesis Inhibition

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Key Words: Endothelial Cells, Apoptosis, Angiogenesis, TUNEL, Orthotopic Tumor

ABSTRACT

We have modified the TUNEL (terminal deoxynucleotidyl transferase uridine nick end labeling) method to permit the detection of apoptotic endothelial cells in tissue sections. Our method combines anti-CD31-mediated immunofluorescence detection of endothelial cells with a TUNEL method that catalytically incorporates fluorescein-dUTP. The diversity and reproducibility of this technique is demonstrated in an orthotopic bladder cancer model following therapy with anti-epidermal growth factor receptor (C225) and in a colon cancer liver metastases model using SU5416, a specific inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) tyrosine kinase. The double labeling method provides an accurate quantification of endothelial cell death, which is a critical endpoint for anti-angiogenic therapies.

INTRODUCTION

Angiogenesis is the dynamic process of new blood vessel formation from established vasculature(1). This process occurs in physiologic situations such as embryonic development and wound healing, and is a key process in tumor progression and metastasis(2). Thus, inhibition of angiogenesis offers an approach to cancer treatment(2-6). Although endothelial cells are accessible to blood-borne agents they do not develop drug-resistant phenotypes(6). Recent work has shown that angiogenic growth factors (VEGF, bFGF) act in part by suppressing apoptosis in endothelial cells, such that interference with their signaling can lead to cell death(7; 8). Induction of apoptosis in endothelial cells, therefore, could be useful for eradication of neoplasms(2; 9).

Tumor endothelial cells are routinely identified by immunohistochemistry using antibodies to endothelial surface antigens (CD31)(10). We sought to develop a dual-

labeling method for simultaneous detection of endothelial cells by CD31 staining and apoptosis-associated DNA fragmentation (by TUNEL analysis). Such detection is not feasible using standard colormetric immunohistochemical development strategies but recent advances in image analysis software allow for fluorescence-based, multi-color immunohistochemical staining. To this end we have modified standard colormetric methods for CD31 immunohistochemistry and TUNEL to allow for dual fluorescence staining of frozen and paraffin-embedded tumor tissues. The feasibility of the method is demonstrated using human umbilical vein endothelial cells undergoing apoptosis *in vitro* and using tumor tissue obtained from human cancers growing in nude mice following treatment with anti-angiogenic agents.

MATERIALS AND METHODS

***In vitro* Model of Endothelial Cell Apoptosis**

Human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured on 1% gelatin coated glass chamber slides in Dubelcco's Modified Eagle Medium (DMEM) supplemented with 15% Fetal Bovine Serum (FBS) and 10 ng/ml basic Fibroblast Growth Factor (bFGF), plus sodium pyruvate, non-essential amino acids, L-glutamine, a 2-fold vitamin solution, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 16 hours, the medium was removed and replaced with the DMEM medium lacking bFGF. Cells (1 x 10^5) were treated with 10 ng/ml mouse anti-human KDR antibody (clone 6.64, ImClone Systems, Inc., New York, NY) for 8 hours. Following treatment, cells were washed with phosphate buffered saline (PBS) and prepared for immunofluorescence double staining.

Immunofluorescence Double Staining and Quantification of Apoptotic Human

Endothelial Cells *in vitro*

HUVEC cells were fixed in 4% paraformaldehyde (methanol-free) for 20 minutes at 4°C.

Cells were washed with PBS once for 5 minutes and permeabilized with 0.2% Triton X-

100 for 15 minutes at room temperature. Samples were washed 3 times with PBS and

incubated with protein blocking solution containing 5% normal horse serum and 1%

normal goat serum in PBS for 20 minutes at room temperature. Blocking solution was

drained and the samples were incubated with a 1:100 dilution of a monoclonal mouse

anti-human CD31 antibody (Dako, Glostrup, Denmark) for 24 hours at 4°C. Samples

were rinsed with PBS containing 0.1% Brij for 5 minutes and 2 times with PBS for 5

minutes. Avoiding exposure to light, samples were incubated with a 1:200 dilution of

Texas Red-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch

Laboratory, Inc., West Grove, PA) for 1 hour at room temperature. Samples were

washed 2 times with PBS containing 0.1% Brij and washed once with PBS for 5 minutes

each. TUNEL was performed according to the manufacturers protocol(Promega,

Madison, WI). Quantification of apoptotic endothelial cells was expressed as an average

of the ratio of apoptotic endothelial cells to the total number of endothelial cells in 3

random fields.

Animals

Male athymic nude mice (BALB/c background) were purchased from the Animal

Production Area of the National Cancer Institute-Frederick Cancer Research and

Development Center (Frederick, MD). The mice were housed and maintained in laminar

flow cabinets under specific pathogen-free conditions in facilities approved by the

American Association for Accreditation of Laboratory Animal Care and in accordance

with current regulations and standards of the U.S. Department of Agriculture, U. S. Department of Health and Human Services, and the National Institutes of Health. The mice were used in accordance with institutional guidelines when they were 8 to 12 weeks old.

Orthotopic Bladder Cancer Model

Cultures of human bladder cancer 253J-BV (11)(60% to 70% confluent) were prepared for *in vivo* injection(12). Mice were anesthetized, a lower midline incision was made, and viable tumor cells (1×10^6 / 0.05 ml) in Hank's Balanced Salt Solution (HBSS) were injected into the bladder wall(11). The formation of a bulla was sign of a satisfactory injection. The bladder was returned to the abdominal cavity and the abdominal wall closed with a single layer of metal clips. Mice were treated with C225 (i.p.) starting 4 weeks after implantation, 2 times/week for 4 weeks. The animals were necropsied approximately 8 weeks later. The primary tumors were removed and quickly frozen in liquid nitrogen using OCT compound (Sakura Finetek, Inc., Torrance, CA). Tissue sections were cut and processed for immunofluorescent analysis.

Colon Cancer Liver Metastasis Model

The CT-26 murine colon cancer cells (10^4) were injected into the spleen of BALB/c nude mice to generate liver metastases(13). After 4 days, 15 mice/group were treated daily (i.p.) with either vehicle alone or a specific TK inhibitor (SU5416 at 12 mg/kg). After 20 days of therapy, livers were weighed as a measure of tumor burden. Livers were either quickly frozen in liquid nitrogen using OCT compound or fixed in 10% formalin and paraffin embedded. Tissue sections were cut and processed for immunofluorescence double staining analysis.

Immunofluorescence Double Staining and Quantification of Apoptotic Endothelial Cells *in situ*

Frozen tissue sections (8 μm) were fixed with cold acetone for 5 minutes, acetone plus chloroform (1:1) for 5 minutes, and acetone for 5 minutes. Samples were washed 3 times with PBS and incubated with protein blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 minutes at room temperature. Blocking solution was drained and the samples were incubated with a 1:400 dilution of rat monoclonal anti-mouse CD31 antibody (Pharmingen, San Diego, CA) for 24 hours at 4°C. Samples were rinsed with PBS 3 times for 3 minutes and incubated with protein blocking solution for 10 minutes at room temperature. Avoiding exposure to light, blocking solution was drained and the samples were incubated with a 1:200 dilution of Texas Red-conjugated goat anti-rat secondary antibody (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) for 1 hour at room temperature. Samples were washed 2 times with PBS containing 0.1% Brij and washed with PBS for 5 minutes. TUNEL was performed using a commercial kit (Promega, Madison, WI) with the following modifications. Samples were fixed with 4% paraformaldehyde (methanol-free) for 10 minutes at room temperature. The samples were washed with PBS two times for 5 minutes and then incubated with 0.2% Triton X-100 for 15 minutes at room temperature. The samples were washed with PBS 2 times for 5 minutes and incubated with equilibration buffer (from kit) for 10 minutes at room temperature. The equilibration buffer was drained and reaction buffer containing equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase (TdT) enzyme was added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 hour, avoiding

exposure to light. The reaction was terminated by immersing the samples in 2X SSC for 15 minutes. Samples were washed 3 times for 5 minutes to remove unincorporated fluorescein-dUTP. For quantification of endothelial cells, the samples were incubated with 300 μg/ml of Hoechst stain for 10 minutes at room temperature. The samples were then washed with PBS 2 times for 5 minutes. Prolong solution (Molecular Probes, Eugene, OR) was used to mount coverslips. Immunofluorescence microscopy was performed using a 40X objective (Zeiss Plan-Neofluar) on an epifluorescence microscope equipped with narrow bandpass excitation filters mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY) to individually select for green, red, and blue fluorescence. Images were captured using a cooled CCD camera (Photometrics, Tucson, AZ) and SmartCapture software (Digital Scientific, Cambridge, England) on a Macintosh computer. Images were further processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA). Endothelial cells were identified by red fluorescence and DNA fragmentation was detected by localized green and yellow fluorescence within the nucleus of apoptotic cells. Quantification of apoptotic endothelial cells was expressed as an average of the ratio of apoptotic endothelial cells to the total number of endothelial cells in 3 random fields.

Immuonfluorescence Double Staining in Formalin-Fixed Paraffin Embedded Sections

Formalin-fixed, paraffin embedded sections (5 μm) were deparaffinized in xylene, rehydrated in grated alcohol, and transferred to PBS. Antigen-retrieval was performed with 200 μg/ml proteinase-K for 30 minutes at room temperature. Samples were washed three times in PBS and double staining was performed according to the protocol

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described above continuing with the protein-blocking step.

RESULTS AND DISCUSSION

The feasibility of dual CD31 and TUNEL staining was first tested using cultures of human endothelial cells. HUVEC cells were induced to undergo apoptosis following VEGF-R blockade using an anti-KDR antibody (human-homolog of Flk-1). Immunofluorescence double staining with the anti-CD31 antibody and TUNEL revealed efficient labeling of HUVEC cells (red fluorescence) and TUNEL positive cells (green and yellow fluorescence) (Figure 1). The treatment group had 65% apoptotic cells versus 7% apoptotic cells in the control group. Parallel analysis of DNA fragmentation by propidium iodide staining and FACS analysis yielded similar results (data not shown).

For the *in vivo* analyses, we used two anti-angiogenic compounds that specifically target endothelial cells of the tumor vasculature. Recently, Perrotte et al.(11) reported the therapy of human 253JBV transitional cell carcinoma (TCC) using a monoclonal antibody mAb C225 directed against the EGF-R. C225 inhibited tumor growth and metastasis by inhibiting expression of several angiogenic factors and inducing apoptosis within the regressing tumors(11). We treated this orthotopic bladder cancer model with C225 antibody and found that the therapy also induced apoptosis of endothelial cells in the tumor vasculature. The endothelial cells were identified by red fluorescence and DNA fragmentation was detected by green fluorescence within the nucleus of apoptotic cells. Superimposing the two images permitted the identification of apoptotic endothelial cells that were identified by red, green, and yellow localized fluorescence(Figure 2).

Vascular endothelial growth factor (VEGF) is a major proangiogenic molecule (14; 15). Over expression of VEGF and its endothelial cell receptor (Flk-1/KDR) has been

associated with metastasis of human colon cancer, and withdrawal of VEGF leads to endothelial cell apoptosis *in vitro* (Figure 1)(9; 16; 17). To confirm this effect under *in vivo* conditions, we treated mice with human colon cancer liver metastases with SU5416 (inhibitor of Flk-1/KDR) and determined whether this treatment induced endothelial cell apoptosis in the liver metastases. The data shown in Figure 3 demonstrate that the technique successfully identified apoptotic endothelial cells in the tumor vasculature.

This double labeling immunofluorescence technique permits quantification of endothelial cells undergoing apoptosis relative to viable endothelial cells and either viable or apoptotic tumor cells. For quantification of apoptotic endothelial cells (Table 1), exact morphology of endothelial cell nuclei was determined by using Hoechst stain (blue fluorescence, not shown) and superimposing the three captured images (CD31, TUNEL, total nuclei). In each of the treatment groups the number of apoptotic endothelial cells was significantly elevated compared to controls. In addition, this method simultaneously permits the identification and quantification of apoptotic tumor cells (also shown in Table I).

In summary, the double labeling immunofluorescence technique is most useful in identifying apoptotic endothelial cells in tissue sections. In addition, our technique permits the identification and quantification of apoptotic tumor cells in the same tissue section, yielding an estimation of total cell toxicity. Thus, the direct effects of anti-cancer therapies on specific cell death can be evaluated. Furthermore, the use of an immunofluorescence-based antibody detection system combined with a fluorescent TUNEL assay provides far more sensitivity in detecting and localizing DNA fragmentation. Finally, because little is known about the other effects of anti-angiogenic

therapies (*i.e.,* growth arrest, inhibition of cytokine production), the versatility of immunofluorescently labeling endothelial cells can be exploited using other protein markers which may provide further insight into the molecular mechanisms underlying their effects. To this end, we have successfully double-labeled endothelial cells with CD31 and PCNA, Bax, Fas, FasL, or Bcl-2.

ACKNOWLEDGEMENTS

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Table I

Effect of treatment with C225 and SU5416 on endothelial and tumor cell apoptosis *in vivo*.

Group	Endothelial Cell Apoptosis[1]	Tumor Cell Apoptosis[2]
Bladder Model		
Control	0%	3.6
C225 therapy	29.8%	11.0
Liver Model		
Control	0.5%	1.6
SU5416 therapy	38.0%	16.3

[1] Quantification of apoptotic endothelial cells is expressed as an average of the ratio of TUNEL positive endothelial cells to the total number of endothelial cells in 3 random fields.

[2] Quantification of apoptotic tumor cells is expressed as an average of the absolute number of TUNEL positive cells in 3 random fields.

FIGURE LEGENDS

Figure 1

Qualitative analysis of immunofluorescence double staining of HUVEC cells *in vitro*.

HUVEC cells are identified by anti-CD31 staining (red fluorescence). Cells induced to

undergo apoptosis with anti-KDR antibody show TUNEL positive staining (localized

green and yellow fluorescence).

Figure 2

Representative immunofluorescence double staining of apoptotic endothelial cells as a

result of C225 therapy in a bladder cancer model (frozen tissue sections). Endothelial

cells of the tumor vasculature are represented by red fluorescence in the CD31 panel.

TUNEL positive cells are represented by green fluorescence. The overlay panel indicates

which endothelial cells are apoptotic (localized red, green, and yellow fluorescence).

Figure 3

Representative immunofluorescence double staining of apoptotic endothelial cells as a

result of SU5416 therapy in a colon cancer liver metastases model (paraffin tissue

sections). Endothelial cells of the tumor vasculature are represented by red fluorescence

in the CD31 panel. TUNEL positive cells are represented by green fluorescence. The

overlay panel indicates which endothelial cells are apoptotic (localized red, green, and

yellow fluorescence).

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PS-341: A NEW AGENT WITH ACTIVITY IN PROSTATE AND PANCREATIC CANCER.

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The proteasome is a multisubunit protease complex that mediates the degradation of a number of proteins involved in cell cycle regulation and survival. The dipeptide boronate, PS-341, is a potent dipeptide boronate inhibitor of the proteasome that was developed by ProScript, Inc. for use in cancer therapy. Preliminary work demonstrated that PS-341 triggers apoptosis in a variety of human pancreatic cancer cell lines, including those engineered to overexpress the anti-apoptotic protein, BCL-2. We therefore conducted studies in nude mice bearing orthotopic human pancreatic tumors to determine the drug's efficacy in vivo.

METHODS: Human PC-3 prostatic or Mia-PaCa-2 pancreatic adenocarcinoma cells (1×10^6) were implanted orthotopically in nude mice. After 7-14 days, animals were treated with various doses of PS-341 (0.3-1 mg/kg i.v., once or twice weekly) for up to 4 weeks. Tumors were harvested, and proliferation, apoptosis, and angiogenesis endpoints were analyzed in paraffin sections by immunohistochemistry.

RESULTS: Treatment of established tumors with PS-341 resulted in central tumor necrosis associated with increased apoptosis and decreased proliferation. Analysis of tumor vascularity by anti-CD31 immunohistochemistry revealed a marked, dose-dependent reduction in microvessel density associated with decreased expression of vascular endothelial growth factor (VEGF). The effects of PS-341 were associated with inhibition of the transcription factor, NFκB, and parallel experiments with a molecular inhibitor of NFκB (IκBαM) confirmed that it was required for VEGF expression.

CONCLUSIONS: PS-341 is effective in established orthotopic human tumors. Its effects involve induction of tumor cell apoptosis and suppression of angiogenesis, and its mechanism of action involves inhibition of NFκB. A Phase I trial of PS-341 in patients with advanced solid tumors is currently underway at our institution.

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- ☒ Animal models for drug evaluation
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- ○ Metastasis and invasion targets (e.g., MMP inhibitors, adhesion)
- ○ Methylation
- ○ Monoclonal antibodies and conjugates
- ○ New extracellular drug targets
- ○ New intracellular drug targets
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- ○ Pharmacogenetics and drug metabolism
- ○ Phase I studies - general
- ○ Phase I studies with molecular endpoints
- ○ Phase II studies - general
- ○ Phase II studies with molecular endpoints
- ○ Phosphokinase targets
- ○ Preclinical pharmacokinetics/ pharmacodynamics
- ○ Radiation interactive agents
- ○ Signal transduction/signaling targets
- ○ Structure-based drug design
- ○ Supportive care agents
- ○ Targets in medically underserved populations
- ○ Telomeres and chromosomal instability
- ○ Toxicity modulators
- ○ Toxicology
- ○ Tubulin-interacting agents
- ○ Other